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(57) Abstract

Novel aprotinin analogues having a selected inhibition profile against serine proteases.

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Aprotinin Analogues and a Process for the Production Thereof

FIELD OF THE INVENTION

The present invention relates to novel aprotinin analogues and to a process for their production.

BACKGROUND OF THE INVENTION

Throughout the present specification the term naturally occuring amino acid (or amino acid residue) refers to one of the α-amino acids which are listed below together with the symbols used to designate the individual amino acid residues:

Asp	Aspartic acid	Ile	Isoleucine
Thr	Threonine	Leu	Leucine
Ser	Serine	Tyr	Tyrosine
Glu	Glutamine acid	Phe	Phenylalanine
Pro	Proline	His	Histidine
Gly	Glycine	Lys	Lysine
Ala	Alanine	Arg	Arginine
Cys	Cysteine	Trp	Tryptophane
Val	Valine	Gln	Glutamine
Met	Methionine	Asn	Asparagine

All amino acids (or amino acid residues) mentioned in the present specification have the L-configuration, except glycine which has no chiral center.

Aprotinin (bovine pancreatic trypsin inhibitor, BPTI) is a polypeptide present in several bovine organs and tissues, such as lymph nodes, pancreas, lung, parotid gland, spleen and liver. It is a single chain polypeptide consisting of 58 amino acid residues cross-linked by three disulphide bridges in the following sequence:

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Arg-Pro-Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-Gly-Pro-Cys-
1   2   3   4   5   6   7   8   9   10  11  12  13  14
Lys-Ala-Arg-Ile-Ile-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-
15  16  17  18  19  20  21  22  23  24  25  26  27  28
Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-Cys-Arg-Ala-Lys-Arg-
29  30  31  32  33  34  35  36  37  38  39  40  41  42
Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-
43  44  45  46  47  48  49  50  51  52  53  54  55  56
Gly-Ala
57  58
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The three disulphide bridges are situated between Cys(5)-Cys(55), Cys(14)-Cys(38) and Cys(30)-Cys(51), respectively.

Aprotinin inhibits various serine proteases, such as trypsin, chymotrypsin, plasmin and kallikrein and is used for the treatment of acute pancreatitis, various states of shock syndroms, hyperfibrinolytic haemorrhage, and myocardial infarction. Administration of aprotinin in high doses significantly reduces blood loss in connection with cardiac surgery.

Aprotinin is extracted from various bovine organs or tissues, such as lung, pancreas and parotid glands. Extraction from animal tissues is a cumbersome process and requires large amounts of the bovine organ or tissue. Aprotinin may also be produced by recombinant DNA-technology by insertion of a gene coding for aprotinin in a suitable microorganism which when cultured in a suitable nutrient medium produces the desired product.

Production of aprotinin analogues in E. coli is described in EP published patent application No. 238,993 and production of aprotinin in yeast is described in Danish patent application No. 4501/87.

Certain aprotinin analogues and derivatives have been described, see for instance Jering H. and Tschesche H., Eur.J.Biochem. 61 (1976), 453-463 describing replacement of Lys(15) with Arg, Phe or Trp or US patent specification No. 4,595,674 describing aprotinin analogues in which the lysine residue in position 15 in the active center of the aprotinin has been replaced by Gly, Ala, Val, Leu, Ile, Met, Arg, L-α-amino butyric acid, L-norvaline, L-norleucine, dehydroalanine or L-homoserine. Also, the above mentioned EP No. 238,993 describes aprotinin analogues having Lys(15) substituted by Arg, Val, Ile, Leu, Phe, Gly, Ser, Trp, Tyr or Ala and/or Met(52) substituted by Glu, Leu, Val, Thr or Ser.

The known aprotinin analogues are claimed to have modified effects and efficacies towards different proteinases. For instance aprotinin(15Val) has a relatively high selectivity for granulocyte elastase and an inhibition effect on collagenase, aprotinin(15Ala) has only a weak inhibitory effect on elastase and aprotinin(15Gly) has an outstanding antitrypsin activity and surprisingly inhibits kallikrein.

SUMMARY OF THE INVENTION

Is is the purpose of the present invention to provide novel aprotinin analogues having a more specific inhibitory effect towards certain serine proteases, such as elastase, kallikrein, t-PA, urokinase and coagulation factors, such as thrombin.

The present invention is based on the surprising fact that replacement of Arg in position 17 of aprotinin(3-58;42Ser) with Ala or of Arg in position 17 with Ala and of Ile in position 19 of aprotinin (3-58;42Ser) with Glu gives rise to a substantial increase in the inhibition of human plasma kallikrein. This is even more pronounced on replacement of Lys in position 15 with Arg and of Arg in position 17 with Ala in aprotinin (3-58; 42Ser).

Accordingly, the present invention is related to aprotinin analogues in which at least one of the amino acid residues 16 to 19 have been replaced by another naturally occuring amino acid residue.

In the above mentioned Danish patent application No. 4501/87 aprotinin analogues with certain amino acid residue substitutions and/or deletions are described. The aim of these amino acid residue substitutions and/or deletions is to avoid proteolytical cleavage at certain basic amino acid residues during the production of the aprotinin analogue in yeast. In particular amino acid residues 1 and 2 may be deleted and one of the basic amino acid residues at the dibasic sequence 41 - 42 may be replaced by another amino acid residue. It has been shown that such aprotinin analogues are produced in high yields in yeast and exhibit the same characteristics as native aprotinin.

To ensure high production in yeast the present aprotinin analogues may further be modified in a similar way. Accordingly the present aprotinin analogues besides being modified in the sequence from amino acid 16 to 19 may also be modified at sequence 1 - 2 and 41 - 42, respectively provided that such further modification does not have an adverse effect on the goal of the present invention.

The present aprotinin analogues may furthermore be modified in position 15 by insertion at this position of another naturally occuring amino acid residue including the known substitutions with naturally occuring amino acid residues.

Native aprotinin contains a disulphide bridge between Cys(14) and Cys(38) which is strongly involved in the tertiary structuring around the active site at Lys(15). This disulphide bond may be split by reducing agents. A more convenient way to avoid having a disulphide bridge at this position in the molecule would be to substitute the cysteine residues with other residues or simply to delete these residues. Accordingly, the present aprotinin analogues may furthermore be modified so that they do not contain a disulphide bridge between Cys(14) and Cys(38). This might impart further interesting characteristics to the molecule.

Finally modifications at positions 12 and 13 might be considered.

Accordingly in its broadest aspect the present invention relates to aprotinin analogues being modified in the sequence from amino acid residue 12 to 19 provided that if Lys(15) has been replaced by another amino acid then at least one further amino acid residue in said sequence has

been substituted or deleted. The present aprotinin analogues may furthermore as described above be modified in the known way at the sequences 1 - 2 and 41 - 42.

According to a further aspect of the present invention there is provided a method for producing the above aprotinin analogues by cultivation of a yeast strain containing a replicable expression vector containing a gene encoding the aprotinin analogues in a suitable nutrient medium followed by recovery of the desired product from the culture medium.

BREIF DESCRIPTION OF THE DRAWINGS

The present invention will be further illustrated by reference to the accompanying drawings in which:

Fig. 1 shows a synthetic gene encoding aprotinin (3-58; 42Ser);

Fig. 2 illustrates the construction of the plasmid pKFN 306;

Fig. 3 illustrates the construction of the plasmid pKFN 504;

Fig. 4 illustrates the construction of the plasmid pMT 636;

Fig. 5A illustrates the inhibition of plasma kallikrein by native aprotinin and by the aprotinin analogues KFN 396 and KFN 399; and

Fig. 5B illustrates the inhibition of plasma kallikrein by native aprotinin and by the aprotinin analogues KFN 396, KFN 772 and KFN 773.

DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS

The present aprotinin analogues may be represented by the following formula (I):

X_1-Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-X_{10}-Arg-Ala-X_{11}-X_{12}-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-Gly-Ala

(I)

in which X_1 is Arg-Pro, Pro or hydrogen, preferably Arg-Pro or hydrogen, most preferred hydrogen; X_2 is any naturally occuring amino acid residue, preferably Gly; X_3 is any naturally accuring amino acid residue, preferably Pro; X_4 and X_{10} are each any naturally ocurring amino acid residue, preferably they are both Cys or both Ala, most preferred they are both Cys;X_5 is Lys, Arg, Val, Thr, Ile, Leu, Phe, Gly, Ser, Met, Trp, Tyr or Ala, preferably Lys or Arg, with the proviso that when X_5 is different from Lys, then at least one of X_2 to X_4 and X_6 to X_9 are different from the amino acid residue at the corresponding position in native aprotinin; X_6 is Ala or Gly, preferably Ala; X_7 is any naturally occuring amino acid residue, preferably Ala or Gly; X_8 is Ile, Leu, Met, Val or Phe, preferably Ile; X_9 is any naturally occuring amino acid residue, preferably Ile or Glu; X_{11} is any naturally occuring amino acid residue, preferably Lys or Arg; X_{12} is Lys, Arg or Ser at least one

of the amino acid residues X_2 to X_9, preferably X_5 to X_9, more preferred X_6 to X_9 being different from the corresponding amino acid residue in native aprotinin.

According to a more narrow aspect the present aprotinin analogues may be represented by the following formula (II):

X_1-Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-Gly-Pro-Cys-X_5-X_6-X_7-X_8-X_9-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-Cys-Arg-Ala-X_{11}-X_{12}-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-Gly-Ala

(II)

in which X_1, X_5, X_6, X_7, X_8, X_9, X_{11} and X_{12} are as defined above for formula (I), at least one of the amino acid residues X_5 to X_9, preferably X_6 to X_9 being different from the corresponding amino acid residue in native aprotinin.

According to an even narrower aspect the aprotinin analogues may be represented by the following formula (III):

X_1-Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-Gly-Pro-Cys-Lys-X_6-X_7-X_8-X_9-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-Cys-Arg-Ala-X_{11}-X_{12}-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-Gly-Ala

(III)

in which X_1, X_6, X_7, X_8, X_9, X_{11} and X_{12} are as defined above for formula (I), at least one of the amino acid residues X_6 to X_9 being different from the corresponding amino acid residue in native aprotinin.

Examples of preferred aprotinin analogues according to the present invention are aprotinin(3-58; 17Ala + 42Ser), which lacks the first two amino acid residues of native aprotinin and has Ala substituted for

Arg in position 17 and Ser substituted for Arg in position 42; aprotinin (3-58; 17Ala + 19Glu + 42Ser), which lacks the first two amino acid residues of native aprotinin and has Ala substituted for Arg in position 17, Glu substituted for Ile in position 19 and Ser substituted for Arg in position 42; and aprotinin (3-58; 15Arg + 17Ala + 42Ser) which lacks the first two amino acid residues of native aprotinin and has Arg substituted for Lys in position 15, Ala substituted for Arg in position 17 and Ser substituted for Arg in position 42, respectively.

Further examples of aprotinin analogues accorrding to the present invention are:

Aprotinin(3-58; 17Ala)
Aprotinin(3-58; 17Ala + 19Glu)
Aprotinin(3-58; 15Arg + 17Ala)
Aprotinin(17Ala + 42Ser)
Aprotinin(15Arg + 17Ala + 42Ser)
Aprotinin(17Ala)
Aprotinin(17Ala + 19Glu)
Aprotinin(15Arg + 17Ala)

For secretion purposes the DNA-sequence encoding the desired aprotinin analogue is fused to a DNA-sequence encoding a signal and leader peptide sequence. The signal and leader peptides are cleaved off by the transformed microorganism during the secretion of the expressed protein product from the cells ensuring a more simple isolation procedure of the desired product. A well suited leader peptide system for yeast is the yeast MFα1 leader sequence or a part thereof (Kurjan, J. and Herskowitz, I., Cell 30 (1982) 933-943). However, any signal- or leader-sequence

which provides for secretion in yeast may be employed and the present invention is not contemplated to be restricted to a specific secretion system.

For expression purposes a promoter sequence is positioned upstream to the DNA-sequence for the desired protein product. Preferably a promoter from a gene indigenous to the yeast host organism is used, e.g. the promoter of the TPI-(triose phosphate isomerase) gene. The DNA-sequence for the desired product will be followed by a transcription terminator sequence, preferably a terminator sequence from a gene indigenous to the host yeast organism, e.g. the terminator of the TPI-gene or the MFα1 gene.

The DNA-sequence encoding the desired aprotinin analogue fused to appropriate promoter, signal, leader and terminator sequences is inserted into an expression vector for expression of the aprotinin analogue in yeast.

The expression vector may be a plasmid capable of independent replication in yeast or capable of integration into the yeast chromosome. The plasmid may preferably be stabilized against plasmid loss by the host microorganism by incorporation of a gene essential for the viability or normal growth of the host cells, e.g. a gene coding for cell division, cell wall biosynthesis, protein synthesis, etc.

Example 1

Aprotinin(3-58; 17Ala + 42Ser)(KFN396)

A sequence encoding aprotinin(3-58; 42Ser) was constructed from a number of oligonucleotides by ligation.

The oligonucleotides were synthesized on an automatic DNA synthesizer using phosphoramidite chemistry on a controlled pore glass support (S.L. Beaucage and M.H. Caruthers (1981) Tetrahedron Letters 22, 1859-1869).

The following 10 oligonucleotides were synthesized:

I: AAAGAGATTTCTGTTTGGAACCTCCATACACTGGTCC
37-mer

II: TTACATGGACCAGTGTATGGAGGTTCCAAACAGAAACT
38-mer

} Duplex A

III: ATGTAAAGCTAGAATCATCAGATACTTCTACAACG
35-mer

IV: CTTGGCGTTGTAGAAGTATCTGATGATTCTAGCT
34-mer

} Duplex B

V: CCAAGGCTGGTTTGTGTCAAACTTTCGTTTACGGTGGCT
39-mer

VI: CTCTGCAGCCACCGTAAACGAAAGTTTGACACAAACCAGC
40-mer

} Duplex C

VII: GCAGAGCTAAGTCCAACAACTTCAAGT
27-mer

VIII: AGCAGACTTGAAGTTGTTGGACTTAG
26-mer

} Duplex D

IX: CTGCTGAAGACTGCATGAGAACTTGTGGTGGTGCCTAAT
39-mer

X: CTAGATTAGGCACCACCACAAGTTCTCATGCAGTCTTC
38-mer

} Duplex E

5 duplexes A - E were formed from the above 10 oligonucleotides as shown in fig. 1 and 2.

20 pmole of each of the duplexes A - E was formed from the corresponding pairs of 5'-phosphorylated oligonucleotides I - X by heating for 5 min. at 90°C followed by cooling to room temperature over a period of 75 minutes. The five duplexes were mixed and treated with T4 ligase. The synthetic sequence was isolated as a 176 bp band after electrophoresis of the ligation mixture on a 2% agarose gel.

The synthetic sequence was ligated to a 330 bp EcoRI-HgaI fragment from plasmid pKFN9 coding for MFα1 signal and leader sequence(1-85) and to the large EcoRI-XbaI fragment from pUC19. The construction of pKFN9 containing a HgaI site immediately after the MFα1 leader sequence is described in European patent application No. 0214826.

The ligation mixture was used to transform a competent E. coli strain (r^-, m^+) selecting for ampicillin resistance.Sequencing of a ^{32}P-XbaI-EcoRI fragment (Maxam, A. and Gilbert, W. (1980) Methods Enzymol. 65, 499-560) showed that plasmids from the resulting colonies contained the correct DNA-sequence for aprotinin(3-58; 42 Ser).

One plasmid pKFN306 was selected for further use. The construction of plasmid pKFN306 is illustrated in fig. 2.

To introduce Ala in position 17 the following oligonucleotides were synthesized as described above:

Ia: CTGGTCCATGTAAAGCTGCTATCATCAGATACTTCTACAACGC
43-mer

IIa: CTTGGCGTTGTAGAAGTATCTGATGATAGCAGCTTTACATGGACCAGTGT
50-mer

The oligonucleotides were 5'-O-phosphorylated by treatment with ATP and T_4-kinase.

A duplex formed by annealing 5'-phosphorylated oligonucleotides Ia and IIa was ligated to the 352 bp EcoRI-PflMI fragment and the 3 kbp EcoRI-StyI fragment, both from pKFN306. pKFN306 encodes the S.cerevisiae mating factor α1 signal-leader (1-85) fused to the synthetic aprotinin (3-58;42 Ser) gene.

The ligation mixture was used to transform a competent E. coli strain (r^-, m^+) selecting for ampicillin resistance. Sequencing of a ^{32}P-XbaI-EcoRI fragment (Maxam, A. and Gilbert, W. (1980) Methods Enzymol. 65, 499-560) showed that plasmids from the resulting colonies contained the correct DNA-sequence for aprotinin(3-58; 17Ala + 42Ser).

One plasmid, pKFN501 was selected for further use. The construction of plasmid pKFN501 is illustrated in fig. 3.

pKFN501 was cut with EcoRI and XbaI and the 0.5 kb fragment was ligated to the 9.5 kb NcoI-XbaI fragment from pMT636 and the 1.4 kb NcoI-EcoRI fragment from pMT636,

resulting in plasmid pKFN504, see fig. 3. Plasmid pMT636 was constructed from pMT608 after deletion of the LEU-2 gene and from pMT479, see fig. 4. pMT608 is described in European patent application No. 195691. pMT479 is described in European patent application No. 163529. pMT479 contains the Schizo. pombe TPI gene (POT), the S. cerevisiae triosephosphate isomerase promoter and terminator, TPI_P and TPI_T (Alber, T. and Kawasaki, G. (1982) J.Mol.Appl.Gen. 1, 419-434). Plasmid pKFN504 contains the following sequence:

TPI_P-MFα1-signal-leader(1-85)-aprotinin(3-58;17Ala + 42Ser)-TPI_T where MFα1 is the S. cerevisiae mating factor alpha 1 coding sequence (Kurjan, J. and Herskowitz, I. (1982) Cell 30, 933-943), signal-leader(1-85) means that the sequence contains the first 85 amino acid residues of the MFα1 signal-leader sequence and aprotinin(3-58; 17Ala + 42Ser) is the synthetic sequence encoding an aprotinin derivative lacking the first two amino acid residues at the N-terminus and having amino acid residues 17 and 42 replaced by an Ala and a Ser residue, respectively.

S. cerevisiae strain MT663 (E2-7B XE11-36 a/α, Δtpi/Δtpi, pep 4-3/pep 4-3) was grown on YPGaL (1% Bacto yeast extract, 2% Bacto peptone, 2% galactose, 1% lactate) to an optical density at 600nm of 0.6.

100 ml of culture was harvested by centrifugation, washed with 10 ml of water, recentrifuged and resuspended in 10 ml of (1.2 M sorbitol, 25 mM Na_2EDTA pH = 8.0, 6.7 mg/ml dithiotreitol). The suspension was incubated at 30°C for 15 minutes, centrifuged and the cells resuspended in 10 ml of (1.2 M sorbitol, 10 mM Na_2EDTA, 0.1 M sodium citrate pH = 5.8, 2 mg Novozym® 234). The suspension was incubated at 30°C for 30 minutes, the cells collected by centrifugation, washed in 10 ml of 1.2 M

sorbitol and 10 ml of CAS (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris HCl (Tris = Tris(hydroxymethyl)-aminomethan) pH = 7.5) and resuspended in 2 ml of CAS. For transformation 0.1 ml of CAS-resuspended cells were mixed with approximately 1 µg of plasmid pKFN504 and left at room temperature for 15 minutes. 1 ml of (20% polyethylenglycol 4000, 10 mM $CaCl_2$, 10 mM Tris HCl, pH = 7.5) was added and the mixture left for further 30 minutes at room temperature. The mixture was centrifuged and the pellet resuspended in 0.1 ml of SOS (1.2 M sorbitol, 33% v/v YPD, 6.7 mM $CaCl_2$, 14 µg/ml Leucine) and incubated at 30°C for 2 hours. The suspension was then centrifuged and the pellet resuspended in 0.5 ml of 1.2 M sorbitol. 6 ml of top agar (the SC medium of Sherman et al., (Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) containing 1.2 M sorbitol plus 2.5% agar) at 52°C was added and the suspension poured on top of plates containing the same agar-solidified, sorbitol containing medium. Transformant colonies were picked after 3 days at 30°C, reisolated and used to start liquid cultures. One such transformant KFN396 was chosen for further characterization.

Yeast strain KFN396 was grown on YPD medium (1% yeast extract, 2% peptone (from Difco Laboratories), and 2% glucose). A 1 liter culture of the strain was shaken at 30°C to an optical density of 600nm of 13. After centrifugation the supernatant was purified by FPLC ion exchange chromatography. The yeast supernatant was filtered through a 0.22 µm Millex® GV filter unit and 1 ml was applied on a MonoS cation exchange column (0.5 x 5 cm) equilibrated with 20 mM Bicine, pH 8.7. After wash with equilibration buffer the column was eluated with a linear NaCl gradient (0-1 M) in equilibration buffer. Trypsin

inhibitor activity was quantified in the eluated fractions by spectrophotometric assay and furthermore by integration of absorption at 280 nm from

$$E^{1\%}_{280}(\text{aprotinin}) = 8.3$$

The yield was about 4.3 mg/liter of aprotinin(3-58; 17Ala + 42Ser).

For amino acid analysis the yeast supernatant (7 ml) was adjusted to pH 8.7 with 0.1 M NaOH and filtered (0.22 µm). The effluent from a Q-Sepharose anion exchange column (1 x 4 cm) equilibrated with 20 mM Bicine, pH 8.7 was applied to a MonoS cation exchange column (0.5 x 5 cm). The cation exchange chromatography was carried out as described above. Concentration of the gradient eluated aprotinin(3-58) was made by rechromatography on MonoS and eluation with steep NaCl-gradient. The collected fractions were further concentrated by vacuum centrifugation to about 100 µl and applied to a RP-HPLC column (Vydac C4, 4.6 x 250 mm). Eluation was carried out with CH_3CN gradient in 0.1% TFA. The collected fractions were concentrated to about 100 µl by vacuum centrifugation and samples were taken for amino acid analysis.

The amino acid analysis appears from the following Table 1. From this table it appears that the product has the expected amino acid composition.

Table 1

Amino acid	Theoretical	Aprotinin (3-58;17Ala + 42Ser) (found)
Asx	5	4.90
Thr	3	2.95
Ser	2	2.10
Glx	3	3.01
Pro	3	3.14
Gly	6	5.93
Ala	7	6.69
Cys	6	5.91
Val	1	1.02
Met	1	0.99
Ile	2	2.00
Leu	2	1.98
Tyr	4	3.73
Phe	4	3.75
Lys	4	4.29
Arg	3	3.21
Total	56	55.60

Example 2

Aprotinin(3-58;17Ala + 19Glu + 42Ser) (KFN399)

A synthetic gene encoding aprotinin(3-58; 17Ala + 19Glu + 42Ser) was constructed as described in Example 1. The following oligonucleotides Ib and IIb were used instead of Ia and IIa:

Ib: CTGGTCCATGTAAAGCTGCTATCGAAAGATACTTCTACAACGC
43-mer

IIb: CTTGGCGTTGTAGAAGTATCTTTCGATAGCAGCTTTACATGGACCAGTGT
50-mer

The pUC19 derived plasmid pKFN503 was constructed in a similar way as pKFN501.

By following the procedure of Example 1 a plasmid pKFN507 was obtained containing the following construction

TPI_P-MFα1-signal-leader(1-85)-aprotinin(3-58;17Ala + 19Glu + 42Ser)-TPI_T

where aprotinin(3-58; 17Ala + 19Glu + 42Ser) is the synthetic gene encoding an aprotinin derivative lacking the first two amino acid residues at the N-terminal and having the residues 17, 19 and 42 of native aprotinin replaced by an alanine, a glutamic acid and a serine residue, respectively.

Plasmid pKFN507 was transformed in yeast strain MT663 as described above and culturing of the transformed strain KFN399 gave about 10 mg/liter of aprotinin(3-58; 17Ala + 19Glu + 42Ser).

The amino acid analysis appears from the following Table 2 and confirms the expected amino acid composition.

Table 2

Amino Acid	Theoretical	Aprotinin (3-58,17Ala + 19Glu + 42Ser) (found)
Asx	5	4.95
Thr	3	2.83
Ser	2	1.90
Glx	4	4.08
Pro	3	2.98
Gly	6	5.98
Ala	7	6.92
Cys	6	5.06
Val	1	0.99
Met	1	0.86
Ile	1	0.99
Leu	2	1.99
Tyr	4	3.77
Phe	4	3.89
Lys	4	4.07
Arg	3	3.06
Total	56	54.36

Example 3

Aprotinin(3-58;15Arg + 17Ala + 42Ser)(KFN773).

A synthetic gene encoding aprotinin(3-58; 15Arg + 17Ala + 42Ser) was constructed as described in Example 1. The following oligonucleotides Ic and IIc were used instead of Ia and IIa:

Ic: CTGGTCCATGTAGAGCTGCTATCATCAGATACTTCTACAACGC
43-mer

IIc: CTTGGCGTTGTAGAAGTATCTGATGATAGCAGCTCTACATGGACCAGTGT
50-mer

The pUC19 derived plasmid pKFN777 was constructed in a similar way as pKFN501.

By following the procedure of Example 1 a plasmid pKFN807 was obtained containing the following construction

TPI_P-MFα1-signal-leader(1-85)-aprotinin(3-58;15Arg + 17Ala + 42Ser)-TPI_T

where aprotinin(3-58; 15Arg + 17Ala + 42Ser) is the synthetic gene encoding an aprotinin derivative lacking the first two amino acid residues at the N-terminal and having the residues 15, 17 and 42 of native aprotinin replaced by an arginine, an alanine and a serine residue, respectively.

Plasmid pKFN807 was transformed in yeast strain MT663 as described above and culturing of the transformed strain KFN773 gave about 8.5 mg/liter of aprotinin(3-58; 15Arg + 17Ala + 42Ser).

The amino acid analysis is shown in Table 3 and confirms the expected amino acid composition.

Table 3

Amino Acid	Theoretical	Aprotinin (3-58,15Arg + 17Ala + 42Ser) (found)
Asx	5	4.95
Thr	3	2.85
Ser	2	1.81
Glx	3	3.01
Pro	3	3.05
Gly	6	5.92
Ala	7	6.91
Cys	6	5.31
Val	1	1.02
Met	1	0.73
Ile	2	1.41
Leu	2	1.99
Tyr	4	3.80
Phe	4	3.94
Lys	3	2.97
Arg	4	4.24
Total	56	53.91

The slightly lowered content of Ile compared with the theoretical value can most probably be ascribed to incomplete hydrolysis of Ile(18)-Ile(19). This is well known in the art.

Example 4

Inhibition of Serine Proteases from Plasma by Aprotinin(3-58; 17Ala + 42Ser) (KFN396) and Aprotinin(3-58; 17Ala + 19Glu + 42Ser) (KFN399), Aprotinin(3-58;15Arg + 42Ser)(KFN772) and Aprotinin(3-58; 15Arg + 17Ala + 42Ser)(KFN773).

Aprotinin(3-58; 17Ala + 42Ser)(KFN396), aprotinin(3-58; 17Ala + 19Glu + 42Ser)(KFN399) and aprotinin (3-58; 15Arg + 17Ala + 42Ser)(KFN773) were purified as described above. As native, bovine pancreatic aprotinin(1-58) batch B 5029-65 (67000 KIU/mg) from NOVO (Bagsvaerd, Denmark) was used. The concentration was calculated using E_{280}^{nm} = 8.3 and M_r=6500. Human plasma kallikrein was obtained from Sigma (St. Louis, MO), bovine factor Xa was purified according to (H. Nobukazu et al. J.Biochem. 97 (1985)1347-1355), human factor IIa (thrombin) was a gift from Dr. W. Lawson (New York State Dept. of Health, Albany, N.Y.), recombinant human factor VIIa was from NOVO (Bagsvaerd, Denmark) and recombinant human protein Ca was from ZymoGenetics, Inc. (Seattle, WA). Substrate S 2302 (H-D-Pro-Phe-Arg-p-nitroanilide) substrate S2238 (H-D-Phe-Pip-Arg-p-nitroanilide) and substrate S2366 (Glu-Pro-Arg-p-nitroanilide) were from Kabi (Stockholm, Sweden). Substrate FXa-1 (methoxycarbonyl DCH-Gly-Arg-p-nitroanilide) was from NycoMed (Oslo, Norway). The experiments were performed in 100 mM NaCl, 50 mM Tris-HCl 0.01 % Tween80, pH 7.4 at 25°C.

Human plasma kallikrein (3 nM) was incubated with aprotinin (0-20 nM) for 30 minutes in a micro-titer well. Substrate S 2302 (0.6 mM) was added to a final volume of 300 µl, and the rate of nitroaniline generation was

measured at 405 nm by means of a Micro ELISA® Autoreader MR 580 from Dynatech Laboratories. The rate is proportional to the concentration of free enzyme. The inhibition of plasma kallikrein by native aprotinin and the four analogues KFN 396, KFN 399, KFN 772 and KFN 773 is shown in fig. 5A and 5B. With native aprotinin a moderate inhibition was observed. The inhibition was strongly increased by analogues KFN 396 and KFN 399 containing Ala in position 17 (Fig. 5A).

A further increase of the inhibition was obtained with Arg in position 15 (KFN 772); and the strongest inhibition was observed with the analogue (KFN 773) with substitution of both position 17 (Ala) and position 15 (Arg) (Fig. 5B).

The analogues were also tested for inhibition of the amidolytic activity of the serine proteases: bovine factor Xa, human factor IIa, human recombinant factor VIIa and human recombinant protein Ca. The experiments were performed essentially as described for plasma kallikrein only appropriate substrates were used. Finally the analogues were analysed for an effect on the coagulation factors of human plasma by means of two clotting tests. These tests, the prothrombin time (PTT) and the activated thromboplastin time (APTT) were performed with General Diagnostics® reagents from Organon (Durham, NC) according to the directions given by the manufacturer. The results of the inhibition experiments are summarized in Table 4 which describes the inhibition profile of the four aprotinin analogues. KFN 773 is characterized by an extraordinaryly strong inhibition of human plasma kallikrein, which is ten fold stronger than that of the Arg 15 analogue (KFN 772). A reverse effect is observed with activated protein C. In

this case the relatively strong inhibition obtained by substitution of Lys 15 to Arg is weakened by further substitution of Arg 17 to Ala.

Table 4. Inhibition profile of aprotinin analogues

	K_i *) (nM); Amidolytic activity of serine proteases§					Clot assays	
Product	Plasma kallikrein	FIIa	FVIIa	FXa	Prot.Ca	PTT	APTT
Native Aprotinin	180	-	-	-	400	-	-
KFN 396	12	-	-	-		-	-
KFN 399	12						
KFN 772	1	-	-	1800	10	-	+
KFN 773	0.1	-	-	150	100	-	+

- No inhibition at 1.0 µM aprotinin analogue
+ Prolonged clotting time at 1.0 µM aprotinin analogue
*) Inhibition constants estimated according to the graphical Dixon method (M. Dixon, Biochem. J. 129 (1972)197-202)
§ Substrates: Plasma kallikrein: S2302; FIIa: S2238; FVIIa: Substrate FXa-1; FXa: Substrate FXa-1; Prot. Ca: S2366.

CLAIMS

1. Aprotinin analogues having the formula X_1-Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-X_{10}-Arg-Ala-X_{11}-X_{12}-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-Gly-Ala in which X_1 means Arg-Pro, Pro or hydrogen, X_4 and X_{10} are both Cys or both Ala or both another naturally occuring amino acid residue, X_2 and X_3 are independently any naturally occuring amino acid residue; X_5 is Lys, Arg, Val, Thr, Ile, Leu, Phe, Gly, Ser, Met, Trp, Tyr or Ala with the proviso that when X_5 is different from Lys then at least one of X_2 to X_4 and X_6 to X_9 is different from the amino acid residue at the corresponding position in native aprotinin; X_6 is Ala or Gly; X_7 is any naturally ocurring amino acid residue; X_8 is Ile, Leu, Met, Val or Phe; X_9 is any naturally occuring amino acid residue; X_{11} is any naturally occuring amino acid residue; X_{12} is Lys, Arg or Ser at

least one of the amino acid residues X_2 to X_9 being different from the corresponding amino acid residue in native aprotinin.

2. Aprotinin analogues according to Claim 1, wherein X_1 is Arg-Pro, Pro or hydrogen, preferably Arg-Pro or hydrogen, most preferred hydrogen.

3. Aprotinin analogues according to Claim 1 or 2, wherein X_2 is Gly.

4. Aprotinin analogues according to any of the preceding claims, wherein X_3 is Pro.

5. Aprotinin analogues according to any of the preceding claims, wherein X_4 and X_{10} are Cys.

6. Aprotinin analogues according to any of the preceding claims wherein X_5 is Lys, Arg, Val, Thr, Ile, Leu, Phe, Gly, Ser, Met, Trp, Tyr or Ala, preferably Lys or Arg with the proviso that when X_5 is different from Lys, then at least one of X_2 to X_4 and X_6 to X_9, preferably X_6 to X_9, is different from the amino acid residue at the corresponding position in native aprotinin.

7. Aprotinin analogues according to any of the preceding claims, wherein X_6 is Ala or Gly, preferably Ala.

8. Aprotinin analogues according to any of the preceding claims, wherein X_7 is Ala or Gly, preferably Ala.

9. Aprotinin analogues according to any of the preceding claims wherein X_8 is Ile, Leu, Met, Val or Phe, preferably Ile.

10. Aprotinin analogues according to any of the preceding claims wherein X_9 is Ile or Glu, preferably Ile.

11. Aprotinin analogues according to any of the preceding claims wherein X_{11} is Lys or Arg, preferably Lys.

12. Aprotinin analogues according to any of the preceding claims wherein X_{12} is Lys, Arg o Ser, preferably Arg or Ser.

13. Aprotinin analogue according to claim 1 having the following structure:
Arg-Pro-Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-Gly-Pro-Cys-Lys-Ala-Ala-Ile-Glu-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-Cys-Arg-Ala-Lys-Arg-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-Gly-Ala.

14. Aprotinin analogue according to claim 1 having the following structure:
Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-Gly-Pro-Cys-Lys-Ala-Ala-Ile-Glu-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-Cys-Arg-Ala-Lys-Arg-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-Gly-Ala.

15. Aprotinin analogue according to claim 1 having the following structure:
Arg-Pro-Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-Gly-Pro-Cys-Lys-Ala-Ala-Ile-Glu-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-Cys-Arg-Ala-Lys-Ser-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-Gly-Ala.

16. Aprotinin analogue according to claim 1 having the following structure:
Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-Gly-Pro-Cys-Lys-Ala-Ala-Ile-Glu-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-Cys-Arg-Ala-Lys-Ser-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-Gly-Ala.

17. Aprotinin analogue according to claim 1 having the following structure:
Arg-Pro-Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-Gly-Pro-Cys-Lys-Ala-Ala-Ile-Ile-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-Cys-Arg-Ala-Lys-Arg-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-Gly-Ala.

18. Aprotinin analogue according to claim 1 having the following structure:
Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-Gly-Pro-Cys-Lys-Ala-Ala-Ile-Ile-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-Cys-Arg-Ala-Lys-Arg-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-Gly-Ala.

19. Aprotinin analogue according to claim 1 having the following structure:
Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-Gly-Pro-Cys-Lys-Ala-Ala-Ile-Ile-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-Cys-Arg-Ala-Lys-Ser-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-Gly-Ala.

20. Aprotinin analogue according to claim 1 having the following structure:
Arg-Pro-Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-Gly-Pro-Cys-Lys-Ala-Ala-Ile-Ile-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-Cys-Arg-Ala-Lys-Ser-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-Gly-Ala.

21. Aprotinin analogue according to claim 1 having the following structure:
Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-Gly-Pro-Cys-Arg-Ala-Ala-Ile-Ile-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-Cys-Arg-Ala-Lys-Ser-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-Gly-Ala.

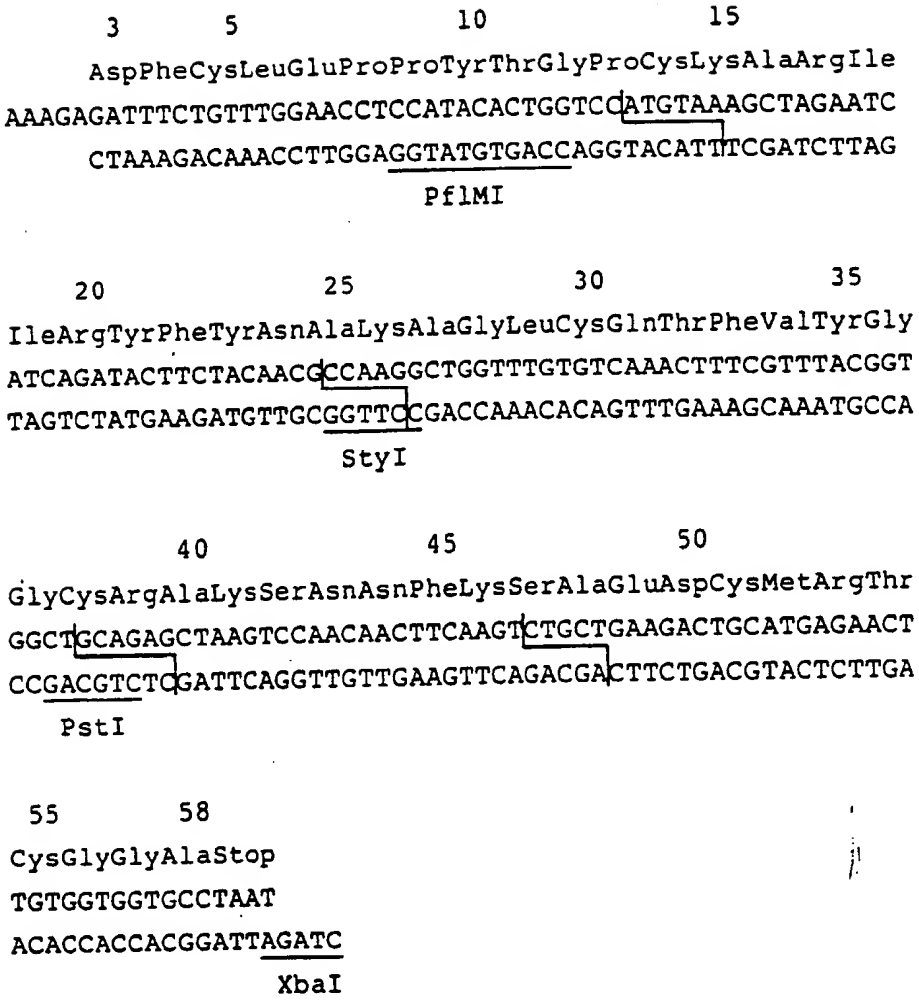
22. Aprotinin analogue according to claim 1 having the following structure:
Arg-Pro-Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-Gly-Pro-Cys-Arg-Ala-Ala-Ile-Ile-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-Cys-Arg-Ala-Lys-Ser-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-Gly-Ala.

23. A process for producing aprotinin analogues according to the general formula (I):

X_1-Asp-Phe-Cys-Leu-Glu-Pro-Pro-Tyr-Thr-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe-Val-Tyr-Gly-Gly-X_{10}-Arg-Ala-X_{11}-X_{12}-Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-Arg-Thr-Cys-Gly-Gly-Ala

(I)

wherein X_1 is Arg-Pro, Pro or hydrogen; X_2 is any naturally occuring amino acid residue, preferably Gly; X_3 is any naturally occuring amino acid residue, preferably Pro; X_4 and X_{10} are any naturally occuring amino acid residues, preferably they are both Ala or both Cys; X_5 is Lys, Arg, Val, Thr, Ile, Leu, Phe, Gly, Ser, Met, Trp, Tyr or Ala, preferably Lys or Arg, with the proviso, that when X_5 is different from Lys, then at least one of X_2 to X_4 and X_6 to X_9 is different from the amino acid residue at the corresponding position in native aprotinin; X_6 is Ala or Gly, preferably Ala; X_7 is any naturally occuring amino acid residue, preferably Ala or Gly; X_8 is Ile, Leu, Met, Val or Phe, preferably Ile; X_9 is any naturally occuring amino acid residue, preferably Ile or Glu; X_{11} is any naturally occuring amino acid residue, preferably Lys or Arg and X_{12} is Lys, Arg or Ser, preferably Arg or Ser, at least one of the amino acid residues X_2 to X_9, preferably X_5 to X_9, more preferred X_6 to X_9 being different from the corresponding amino acid residue in native protein in yeast comprising culturing a yeast strain containing a replicable expression vector, the vector comprising a gene coding for the aprotinin analogue and DNA sequences that allow for the expression of the aprotinin analogue in a suitable nutrient medium and recovering the expressed aprotinin analogues.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CTAAAGACAAACCTTGGAGGTATGTGACCAGGTACATTTCGATCTTAG
PflMI
20 25 30 35
IleArgTyrPheTyrAsnAlaLysAlaGlyLeuCysGlnThrPheValTyrGly
ATCAGATACTTCTACAACGCCAAGGCTGGTTTGTGTCAAACTTTCGTTTACGGT
TAGTCTATGAAGATGTTGCGGTTCCGACCAAACACAGTTTGAAAGCAAATGCCA
StyI
40 45 50
GlyCysArgAlaLysSerAsnAsnPheLysSerAlaGluAspCysMetArgThr
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CCGACGTCTCGATTCAGGTTGTTGAAGTTCAGACGACTTCTGACGTACTCTTGA
PstI
55 58
CysGlyGlyAlaStop
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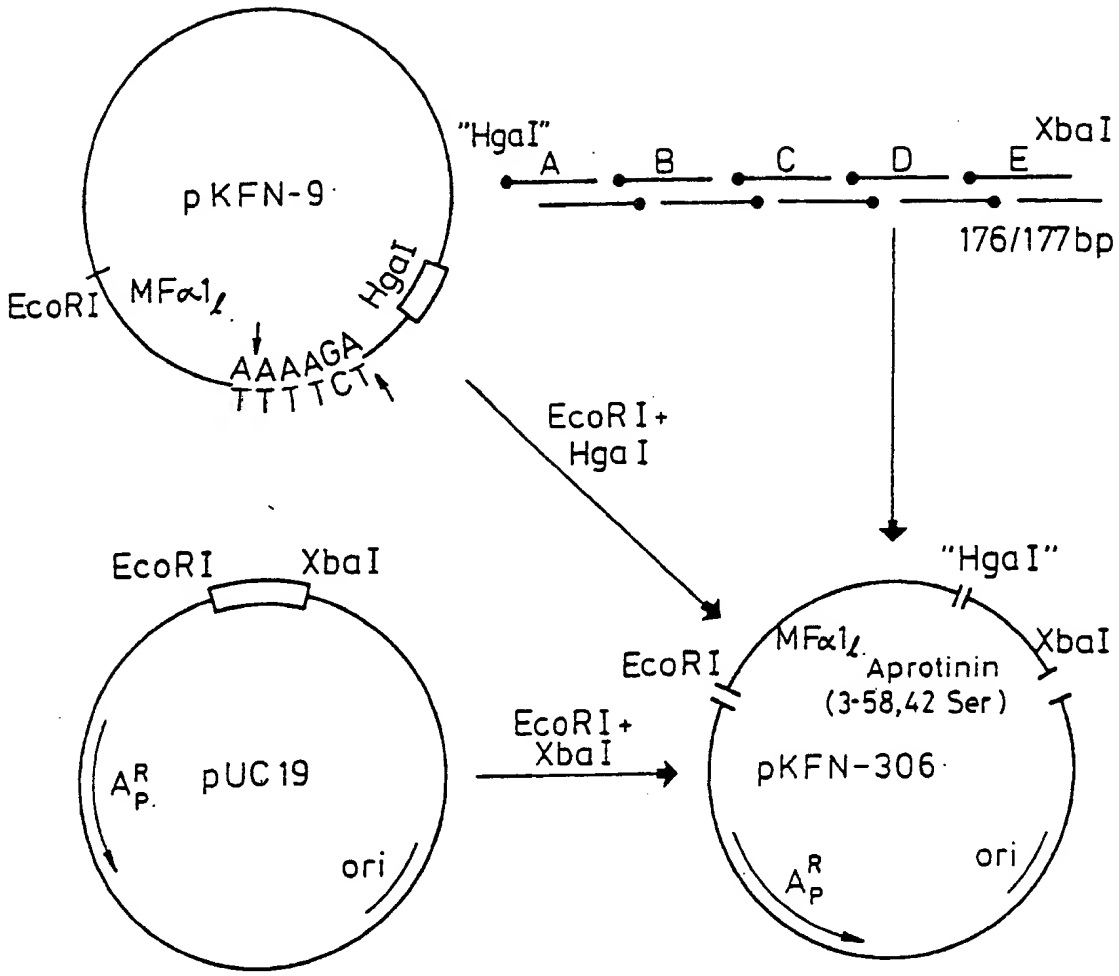
ACACCACCACGGATTAGATC
XbaI

FIG. 1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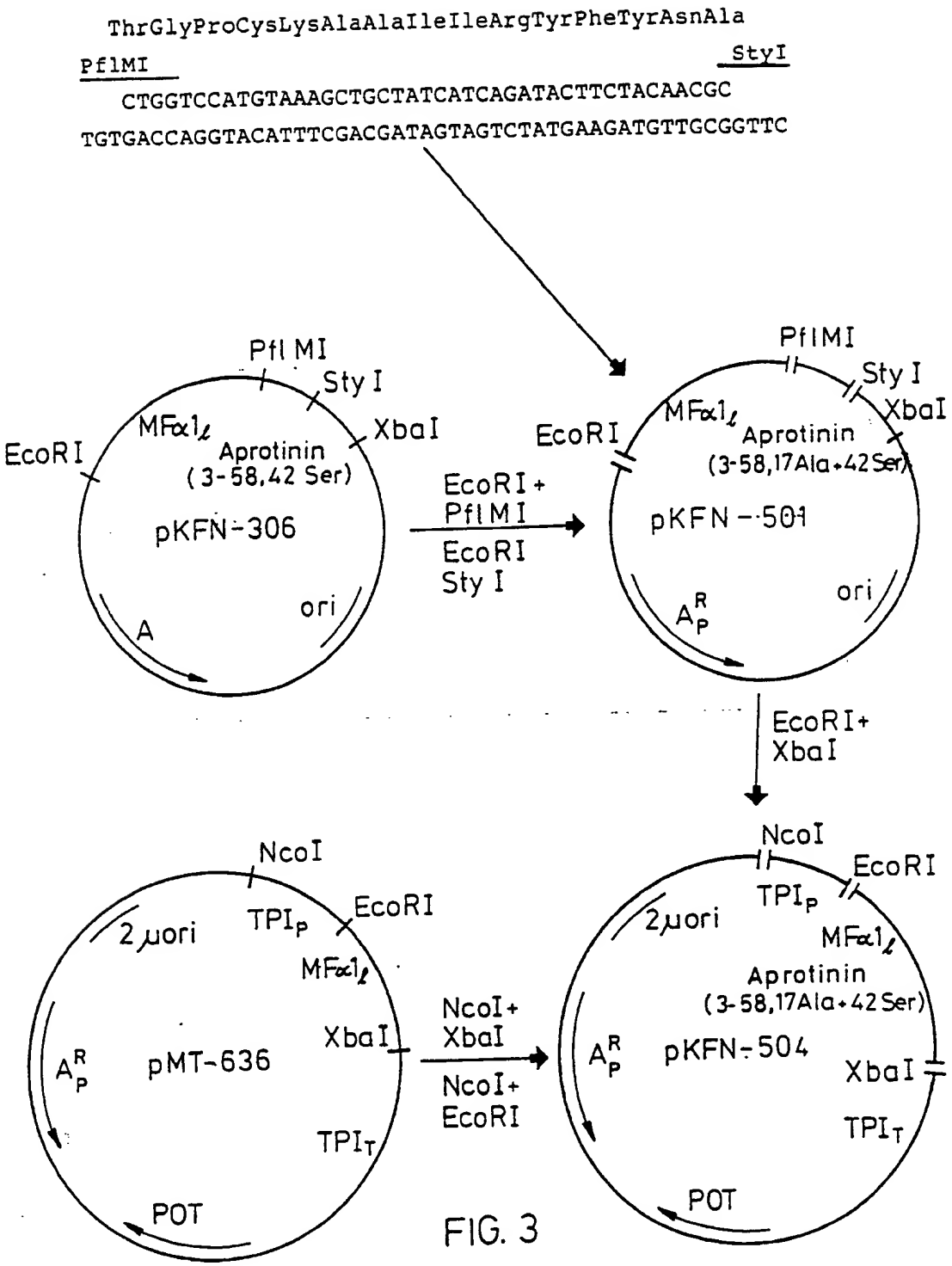
MFα1
HgaI
AAAAGA
TTTTCT
"HgaI"
A
B
C
D
E
XbaI
176/177bp
EcoRI+
HgaI
EcoRI
XbaI
A_P^R
pUC19
ori
EcoRI+
XbaI
"HgaI"
EcoRI
MFα1
Aprotinin
(3-58,42 Ser)
XbaI
pKFN-306
A_P^R
ori

FIG. 2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PflMI
Sty I
EcoRI
MFα1ℓ
Aprotinin
(3-58,42 Ser)
XbaI
pKFN-306
ori
A
EcoRI+
PflMI
EcoRI
Sty I
PflMI
Sty I
XbaI
EcoRI
MFα1ℓ
Aprotinin
(3-58,17Ala+42Ser)
pKFN--501
ori
EcoRI+
XbaI
NcoI
EcoRI
2μori
TPIP
MFα1ℓ
XbaI
pMT-636
TPIT
POT
NcoI+
XbaI
NcoI+
EcoRI
NcoI
EcoRI
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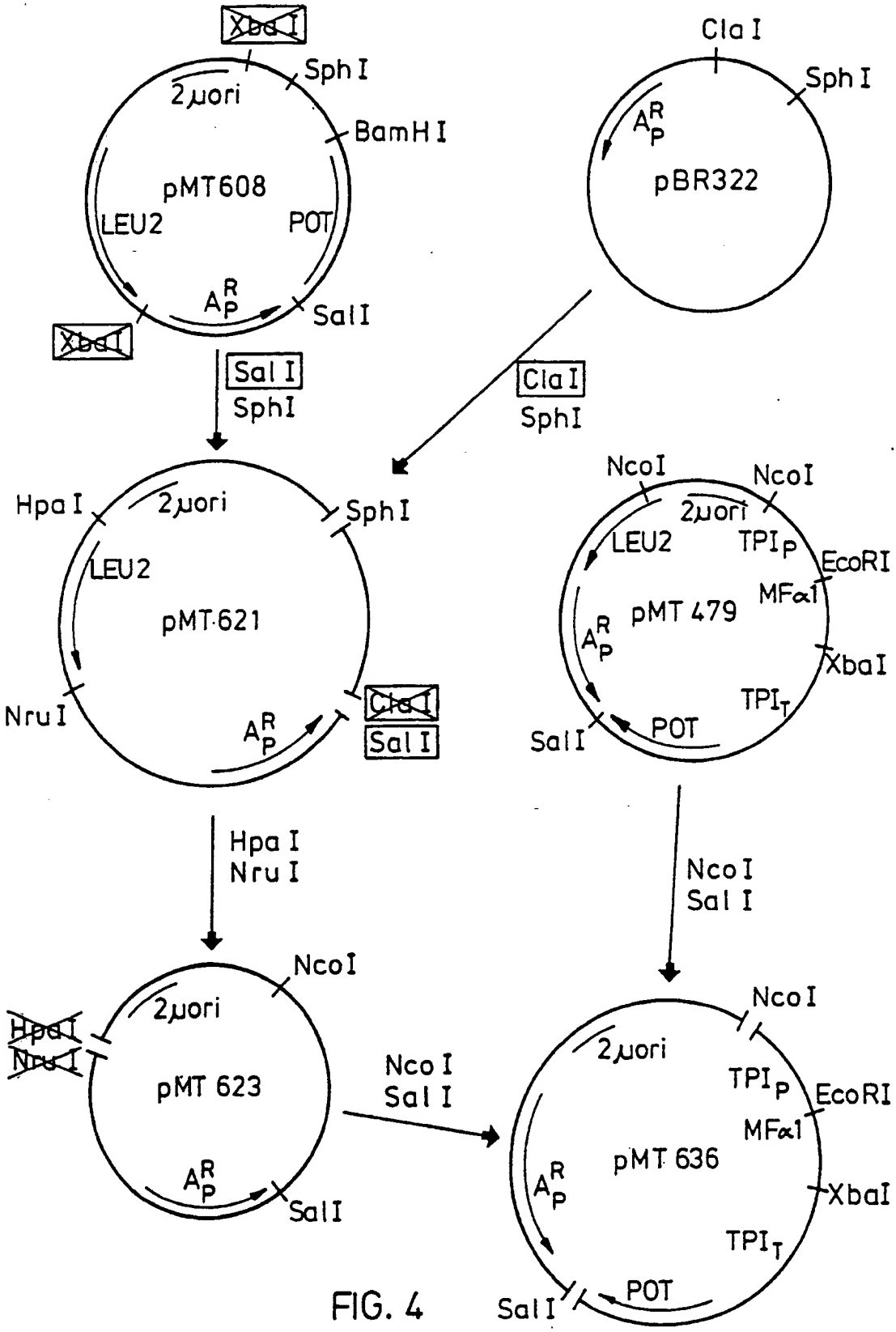
TPIT
POT

FIG. 3


XbaI
SphI
2μori
BamHI
pMT608
LEU2
POT
A_P^R
SalI
XbaI
SalI
SphI
ClaI
SphI
ClaI
SphI
pBR322
A_P^R
HpaI
2μori
SphI
LEU2
pMT621
NruI
A_P^R
ClaI
SalI
NcoI
NcoI
2μori
LEU2
TPI_P
EcoRI
MFα1
pMT 479
A_P^R
XbaI
TPI_T
SalI
POT
HpaI
NruI
NcoI
SalI
NcoI
2μori
HpaI
NruI
pMT 623
A_P^R
SalI
NcoI
SalI
NcoI
2μori
TPI_P
EcoRI
MFα1
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XbaI
TPI_T
SalI
POT

FIG. 4

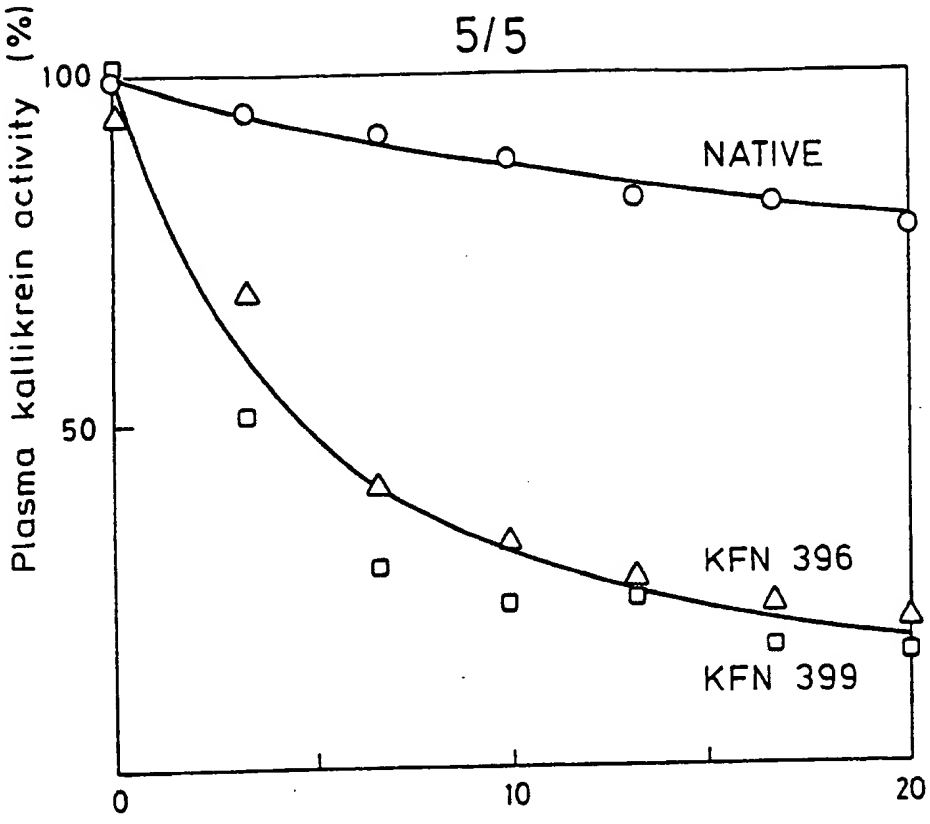
Plasma kallikrein activity (%)
100
50
0
10
20
NATIVE
KFN 396
KFN 399
[Aprotinin] (nM)

FIG. 5A

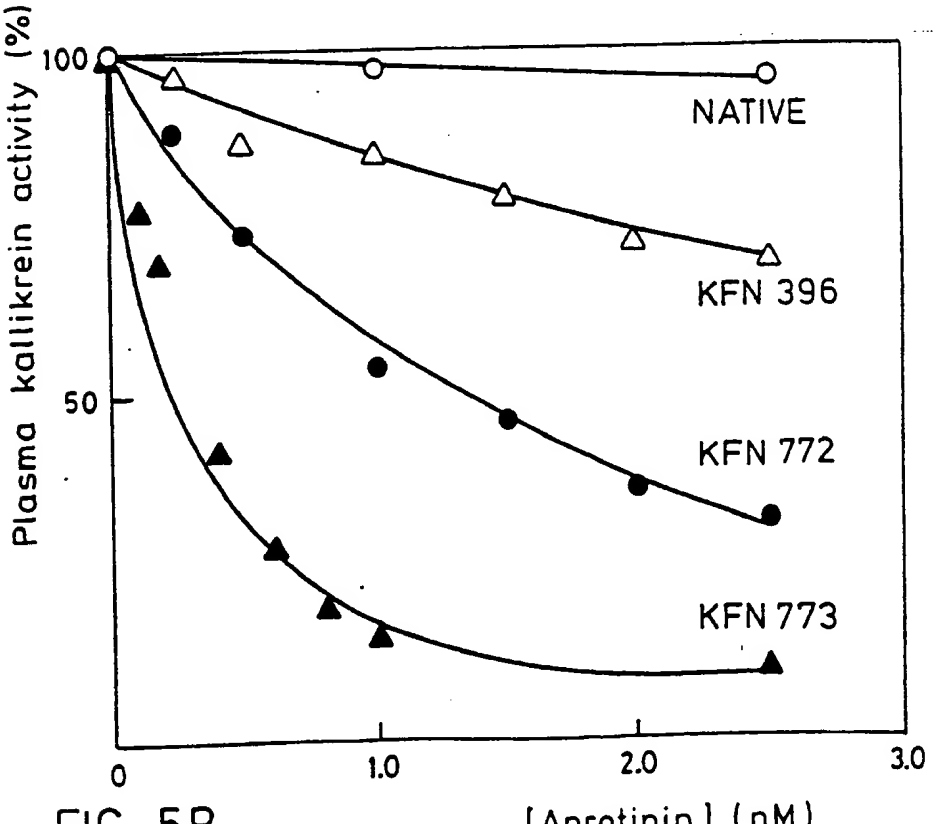
Plasma kallikrein activity (%)
100
50
0
1.0
2.0
3.0
NATIVE
KFN 396
KFN 772
KFN 773
[Aprotinin] (nM)

FIG. 5B

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK89/00096

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC 4

C 07 K 7/10, C 12 N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC 4	C07C 103/52; C07K 7/10
US Cl	260:112.5, 424:177; 514:2, 12; 530:324

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	EP,A2, 0 132 732 (BAYER AG) 13 February 1985 JP, 60056999 US, 4595674 AU, 560584 CA, 1238000 DE, 3471940 DE, 3339693	1-12, 22
Y	EP,A2, 0 238 993 (BAYER AG) 30 September 1987 GB, 2188322 JP, 63007794	1-12, 22 23
Y	EP,A2, 0 244 627 (BAYER AG) 11 November 1987 GB, 2188933 JP,63012299	1-12, 23
P, X	EP,A2, 0 297 362 (BAYER AG) 4 January 1989 DE, 3724570	1-23

.../...

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1989-07-14	1989 -07- 28
International Searching Authority	**Signature of Authorized Officer**
Swedish Patent Office	Elisabeth Carlborg

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS ONSIDERED T BE RELEVANT (C NTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X Y	Biol. Chem. Hoppe-Seyler Vol. 367, pp. 1167-1176, November 1986 Eugen Schnabel et al "[$Ala_2^{14,38}$]Aprotinin:Preparation by Partial Desulphurization of Aprotinin by Means of Raney Nickel and Comparison with Other Aprotinin Derivatives".	1-4 5-12